



SensoLyte[®] Rh110 Cathepsin K Assay Kit

Fluorimetric

Revision Number: 1.1	Last updated: October 2014
Catalog #	AS-72152
Kit Size	100 Assays (96-well plate)

- **Optimized Performance:** This kit detects Cathepsin K activity.
- **Enhanced Value:** Ample reagents to perform 100 assays in a 96-well format.
- **High Speed:** The entire process can be completed in one hour
- **Assured Reliability:** Detailed protocol and references are provided.

Kit Components, Storage and Handling

Component	Description	Quantity
Component A	Rh110 Cathepsin K substrate, Ex/Em=490 /520 nm upon cleavage	1.4 mM, 50 μ L
Component B	Rh110, fluorescence reference standard, Ex/Em=490 nm/520 nm	1.4 mM, 20 μ L
Component C	Procathepsin K, human recombinant	0.15 mg/mL, 10 μ L
Component D	Assay Buffer	20 mL
Component E	Cathepsin K Activation Buffer	100 μ L
Component F	Cathepsin K Inhibitor	100 μ M, 10 μ L
Component G	DTT	1 M, 200 μ L

Other Materials Required (but not provided)

- 96-well microplate: Black microplates provide better signal to noise ratio.
- Fluorescence microplate reader: Capable of detecting Em at 520 nm with Ex at 490 nm.

Storage and Handling

- Store all kit components at -20°C, except for Component C
- Store Component C at -80°C
- Protect Components A and B from light and moisture.

- Component D can be stored at room temperature for convenience.

Introduction

Cathepsin K is a cysteine protease that plays a role in the degradation of protein components of bone matrix during bone resorption.¹ Produced by bone resorbing macrophages and synovial fibroblasts, cathepsin K cleaves proteins such as collagen type I, collagen type II and osteonectin.² It has potential as a drug target in autoimmune diseases and osteoporosis.^{3,4}

The SensoLyte[®] Rh110 Cathepsin K Activity Assay Kit provides a convenient assay for screening of enzyme inhibitors and activators or for continuous assay of enzyme activity using a fluorogenic substrate. Upon cleavage by Cathepsin K, this substrate generates the Rh110 (rhodamine 110) fluorophore which has a bright green fluorescence and can be detected at excitation/emission=490/520 nm. The longer wavelength spectra and higher extinction coefficient of Rh110 provide greater sensitivity and less interference from other reaction components.

Protocol

Note 1: For standard curve, please refer to [Appendix II](#) (optional).

Note 2: Please use Protocol A or B based on your needs.

Protocol A. Screening Cathepsin K inhibitors using purified enzyme.

1. Prepare working solutions.

Note: Warm all kit components until thawed to room temperature before starting the experiments.

1.1 Prepare assay buffer: Prepare fresh assay buffer for each experiment according to Table 1.

Use this DTT-containing assay buffer in all subsequent steps.

Table 1. Assay buffer for one 96-well plate (100 assays)

Components	Volume
Assay buffer (Component D)	9.95 mL
1 M DTT (Component G)	50 μ L
Total volume	10 mL

1.2 Cathepsin K substrate solution: Dilute Cathepsin K substrate (Component A) 1:100 in assay buffer. For each experiment, prepare fresh substrate solution.

Table 2. Cathepsin K substrate solution for one 96-well plate (100 assays)

Components	Volume
Cathepsin K substrate (100X, Component A)	50 μ L
Assay buffer	4.95 mL
Total volume	5 mL

1.3 Prepare Cathepsin K activation buffer: Prepare fresh activation buffer for each experiment according to Table 3.

Table 3. Activation buffer.

Components	Volume
Activation buffer (Component E)	99 μ L
1 M DTT (Component G)	1 μ L
Total volume	100 μ L

1.4 Cathepsin K diluent:

Activation of enzyme: Add 10 μL of activation buffer to the vial (10 μL) of Procathepsin K (Component C) and incubate 40 min. at room temperature. Dilute the activated enzyme 1:300 in assay buffer. This amount of enzyme is enough for a full 96-well plate. If not using an entire plate, adjust the amount of enzyme to be diluted accordingly.

Note: Add an equal volume of activation buffer to the enzyme to activate enzyme. This buffer adjusts the pH to 4.0, thereby activating enzyme. Prepare enzyme diluents immediately before use. Do not vortex the enzyme solution. Prolonged storage or vigorous agitation of the diluted enzyme will cause denaturation. Store on ice.

1.5 Cathepsin K inhibitor: Dilute 100 μM inhibitor solution (Component F) 1:1000 in assay buffer to get a concentration of 100 nM. Add 10 μL of the diluted compound into each of the inhibitor control well.

2. Set up the enzymatic reaction.

2.1 Add test compounds and diluted enzyme solution to the microplate wells. The suggested volume of enzyme solution for a 96-well plate is 40 μL /well and test compound is 10 μL /well.

2.2 Simultaneously establish the following control wells, as deemed necessary:

- Positive control contains the enzyme without test compound.
- Inhibitor control contains Cathepsin K enzyme and inhibitor.
- Vehicle control contains Cathepsin K enzyme and vehicle used in delivering test compound (e.g. DMSO, concentration not to exceed 1%).
- Test compound control contains assay buffer and test compound. Some test compounds have strong autofluorescence and may give false results.
- Substrate control contains assay buffer.

2.3 Using the assay buffer, bring the total volume of all controls to 50 μL .

2.4 Optional: Pre-incubate the plate for 10 min. at assay temperature. Any temperature (*the assay temperature*) from room temperature to 37°C may be used, as long as the subsequent incubations are performed at the same temperature.

3. Run the enzymatic reaction.

3.1 Add 50 μL of Cathepsin K substrate solution into each well. For best accuracy, it is advisable to have the substrate solution equilibrated to the assay temperature. Mix the reagents completely by shaking the plate gently for 30 sec.

3.2 Measure fluorescence signal:

- For kinetic reading: Immediately start measuring fluorescence at Ex/Em=490 nm/520 nm continuously and record data every 5 min. for 30 to 60 min.
- For end-point reading: Incubate the reaction for 30 to 60 min. Keep plate from direct light; then measure fluorescence at Ex/Em=490 /520 nm.

3.3 For methods of data analysis: Refer to Appendix I.

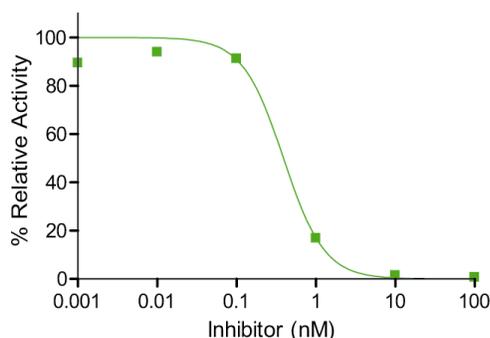


Figure 1. Inhibition of Cathepsin K activity measured using Sensolyte® Rh110 Cathepsin K Assay Kit.

Protocol B. Measuring Cathepsin K activity in biological samples.

1. Prepare Cathepsin K containing biological samples.

1.1 Prepare cell extracts:

Note: According to our data, Cathepsin L can also cleave Rh110 substrate provided in this kit. Since cells or tissues may contain different cathepsins, Cathepsin L activity in your biological sample may need to be inhibited using a specific Cathepsin L inhibitor (Cat# 62935). We recommend using the Sensolyte® 520 Cathepsin K Assay Kit (Cat#72151). This assay provides a more specific Cathepsin K substrate (FRET) for biological samples.

- Use freeze-thaw cycles for cell lysis.
- Suspension cells are collected by centrifugation at 500 X g for 5 min and washed by PBS.
- For adherent cells, aspirate the growth medium, trypsinize, followed by inactivation of trypsin using medium with 10% FBS. Wash cells with PBS before proceeding to the lysis step.
- Add an appropriate amount of assay buffer (see Step 2.1) to cells or cell pellet.
- Perform 3 freeze-thaw cycles to lyse cells.
- Centrifuge cell suspension for 10 min at 13,000X g, 4°C. Collect supernatant and store at -70°C until use.

2. Prepare working solutions.

Note: Warm all kit components until thawed to room temperature before starting the experiments.

2.1 Prepare assay buffer: Prepare fresh assay buffer for each experiment according to Table 1. **Use this DTT-containing assay buffer in all subsequent steps.**

Table 1. Assay buffer for one 96-well plate (100 assays).

Components	Volume
Assay buffer (Component D)	9.95 mL
1 M DTT (Component G)	50 μ L
Total volume	10 mL

2.2 Cathepsin K substrate solution: Dilute Cathepsin K substrate (Component A) 1:100 in assay buffer. For each experiment, prepare fresh substrate solution.

Table 2. Cathepsin K substrate solution for one 96-well plate (100 assays).

Components	Volume
Cathepsin K substrate (100X, Component A)	50 μ L
Assay buffer	4.95 mL
Total volume	5 mL

2.3 Prepare Cathepsin K activation buffer: Prepare fresh activation buffer for each experiment according to Table 3.

Table 3. Activation buffer.

Components	Volume
Activation buffer (Component E)	99 μ L
1 M DTT (Component G)	1 μ L
Total volume	100 μ L

2.4 Cathepsin K diluent:

Activation of enzyme: If using purified Procathepsin K enzyme as a positive control, add an equal volume of activation buffer to the enzyme and incubate 40 min at room temperature. Dilute activated enzyme 1:300 in assay buffer. Adjust the amount of enzyme to be diluted accordingly.

Note: The activation buffer adjusts the pH to 4.0, thereby activating enzyme. Prepare enzyme diluents immediately before use. Do not vortex the enzyme solution. Prolonged storage or vigorous agitation of the diluted enzyme will cause denaturation. Store on ice.

3. Set up enzymatic reaction.

3.1 Add 5-50 μ L of Cathepsin K containing biological sample.

3.2 Simultaneously establish the following control wells, as deemed necessary:

- Positive control contains active Cathepsin K diluents.
- Substrate control contains assay buffer.

3.3 Using assay buffer, bring the total volume of all samples to 50 μ L/well.

3.4 Optional: Pre-incubate the plate for 10 min. at assay temperature. Any temperature (the *assay temperature*) from room temperature to 37°C may be used, as long as the subsequent incubations are performed at the same temperature.

4. Run the enzymatic reaction.

4.1 Add 50 µL of Cathepsin K substrate solution into each well. For best accuracy, it is advisable to have the substrate solution equilibrated to the assay temperature. Mix the reagents completely by shaking the plate gently for 30 sec.

4.2 Measure fluorescence signal:

- For kinetic reading: Immediately start measuring fluorescence at Ex/Em=490/520 nm continuously and record data every 5 min. for 30 to 60 min.
- For end-point reading: Incubate the reaction for 30 to 60 min. Keep plate from direct light; then measure fluorescence at Ex/Em=490/520 nm.

4.3 For methods of data analysis: Refer to Appendix I.

Appendix I. Data Analysis

- The fluorescence reading from the substrate control well is used as the background fluorescence. This background reading should be subtracted from the readings of the other wells containing substrate. All fluorescence readings are expressed in relative fluorescence units (RFU).
- For kinetics analysis:
 - Plot data as RFU versus time for each sample. To convert RFUs to the concentration of the product of the enzymatic reaction, please refer to Appendix II for establishing a fluorescence reference standard.
 - Determine the range of initial time points during which the reaction is linear. Typically, the first 10-15% of the reaction will be the optimal range.
 - Obtain the initial reaction velocity (V_o) in RFU/min by determining the slope of the linear portion of the data plot.
 - A variety of data analyses can be done, e.g., determining inhibition %, EC_{50} , IC_{50} , K_m , K_i , etc.
- For endpoint analysis:
 - Plot data as RFU versus concentration of test compounds.
 - A variety of data analyses can be done, e.g., determining inhibition %, EC_{50} , IC_{50} , etc.

Appendix II. Instrument Calibration

- Rh110 fluorescence reference standard: Dilute 1.4 mM Rh110 (Component B) to 14 μ M in 1X assay buffer. Do 2-fold serial dilutions to get concentrations of 7, 3.5, 1.75, 0.9, 0.45, and 0.22 μ M, include an assay buffer blank. Add 50 μ L/well of these serially diluted Rh110 reference solutions.
- Add 50 μ L/well of the diluted Cathepsin K substrate solution (refer to Protocol A for preparation).

Note: Cathepsin K substrate solution is added to the reference standard to normalize for the intrinsic substrate fluorescence. If multiple concentrations of substrate are used, this step must be performed for each concentration.

- Measure the fluorescence of the reference standard and substrate control wells at Ex/Em=490 nm/520 nm. Use the same setting of sensitivity as used in the enzyme reaction.
- Plot the Rh110 reference standard curve as RFU (relative fluorescent units) versus concentration.
- The final concentrations of Rh110 reference standard are 7, 3.5, 1.75, 0.9, 0.45, 0.22, 0.11, and 0 μ M. This reference standard is used to calibrate the variation of different instruments and different experiments. Since the proteolytic cleavage of the Rh110 substrate consists of two steps, with both the intermediate and final products having fluorescence, the Rh110 reference standard cannot serve as an indicator of the amount of enzymatic reaction final product.

References :

1. Motyckova, G. et al, *PNAS*. **98**, 5798 (2001).
2. Hou, WS. et al, *Am. J. Pathol.* **159**, 2167 (2001).
3. Skoumal, M. et al, *Arthritis Res Ther.* **7**, 65(2005).
4. Asagiri, M. et al, *Science* **319**, 624 (2008).